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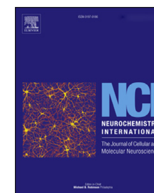

Vascular endothelial growth factor influences migration and focal adhesions, but not proliferation or viability, of human neural stem/progenitor cells derived from olfactory epithelium

Gerardo Bernabé Ramírez-Rodríguez [a, *], Gerardo Rodrigo Perera-Murcia [a, 1], Leonardo Ortiz-López [a], Nelly Maritza Vega-Rivera [b], Harish Babu [c], Maria García-Anaya [d], Jorge Julio González-Olvera [d]

[a] Laboratory of Neurogenesis, Division of Clinical Investigations, National Institute of Psychiatry "Ramón de la Fuente Muñiz", Calz. México-Xochimilco 101, 14370 Ciudad de México, Mexico
[b] Laboratory of Neuropsychopharmacology, Division of Neuroscience, National Institute of Psychiatry "Ramón de la Fuente Muñiz", Calz. México-Xochimilco 101, 14370 Ciudad de México, Mexico
[c] Department of Neurosurgery, Cedars-Sinai Medical Center, 127 S. San Vicente Boulevard, Los Angeles, CA 90048, USA
[d] Division of Clinical Investigations, National Institute of Psychiatry "Ramón de la Fuente Muñiz", Calz. México-Xochimilco 101, 14370 Ciudad de México, Mexico

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ABSTRACT

In humans, new neurons are continuously added in the olfactory epithelium even in the adulthood. The resident neural stem/progenitor cells (hNS/PCs-OE) in the olfactory epithelium are influenced by several growth factors and neurotrophins. Among these modulators the vascular endothelial growth factor (VEGF) has attracted attention due its implicated in cell proliferation, survival and migration of other type of neural/stem progenitor cells. Interestingly, VEGFr2 receptor expression in olfactory epithelium has been described in amphibians but not in humans. Here we show that VEGFr is expressed in the hNS/PCs-OE. We also investigated the effect of VEGF on the hNS/PCs-OE proliferation, viability and migration *in vitro*. Additionally, pharmacological approaches showed that VEGF (0.5 ng/ml)-stimulated migration of hNS/PCs-OE was blocked with the compound DMH4, which prevents the activation of VEGFr2. Similar effects were found with the inhibitors for Rac (EHT1864) and p38MAPK (SB203850) proteins, respectively. These observations occurred with changes in focal adhesion contacts. However, no effects of VEGF on proliferation or viability were found in hNS/PCs-OE. Our results suggest that hNS/PCs-OE respond to VEGF involving VEGFr2, Rac and p38MAPK.

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1. Introduction

Neurogenesis in the olfactory epithelium occurs from resident neural stem cells and continues during adulthood (Graziadei, 1965; Schwob et al., 2017). These new olfactory neurons connect to the mitral cells of the olfactory bulb to influence olfactory memory (Breer et al., 2005; Oka et al., 2006). Several processes such as proliferation and migration are involved in the maturation of neural stem cells to mature olfactory neurons (Hahn et al., 2005; Schwob et al., 2017). The actions of several modulators such as the endothelial growth factor (EGF), fibroblast derived growth factor (FGF), neuronal growth factor (NGF), neurotrophin 3 and 4/5, brain-derived neurotrophic factor (BDNF), insulin growth factor (IGF), platelet-derived growth factor (PDGF), glial-derived neurotrophic factor (GDNF) (Getchell et al., 2000; Mackay-Sima and Chuahb, 2000; Ortiz-Lopez et al., 2017a; Roisen et al., 2001; Zhang et al., 2004) influence the neurogenic process in the olfactory epithelium. Interestingly, it has been suggested that some factors are derived from the capillaries present in the lamina propria

* Corresponding author. Laboratory of Neurogenesis, Division of Clinical Research, National Institute of Psychiatry, Calzada México-Xochimilco No. 101, Colonia San Lorenzo Huipulco, Delegación Tlalpan, Ciudad de México C.P. 14370, Mexico.
E-mail address: gbernabe@imp.edu.mx (G.B. Ramírez-Rodríguez).
[1] Present address: Instituto de Fisiología Celular Neurociencias. Posgrado en Ciencias Bioquímicas. Universidad Nacional Autónoma de México. Circuito Exterior S/N Ciudad Universitaria, México. D.F. 04510. México.

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(Mackay-Sim and Beard, 1987). Among them, VEGF may be a potent candidate to regulate neurogenesis in the human olfactory epithelium.

VEGF has shown interesting effects on different neural precursor cells such as oligodendrocyte precursor cells and adult hippocampal precursor cells (Fournier and Duman, 2012; Hayakawa et al., 2011). Moreover, this factor promotes neurite outgrowth and survival (Jin et al., 2006; Khaibullina et al., 2004) In addition, VEGF acts as a direct stimulator of neurogenesis in the dentate gyrus of the hippocampus (Fabel et al., 2003; Jin et al., 2002), via its receptor, VEGFr2, that, at least in central nervous system, is directly implicated in the pro-neurogenic effects of VEGF (Fabel et al., 2003; Jin et al., 2002; Ruiz de Almodovar et al., 2009). In rodents, VEGF acts through the activation of tyrosine kinase receptors and among these receptors, the VEGFr2 receptor has shown a key role in the pro-neurogenic effects of VEGF (Fournier and Duman, 2012). After binding with VEGFr2, several signaling pathways are activated to transduce VEGF signal, such as, but not limited to, p38MAPK, the small G protein Rac or phospholipase gamma, that activate proteins implicated in cell survival, dendrite formation, cellular migration or proliferation (i.e. Carretero-Ortega et al., 2010; Fournier and Duman, 2012). Thus, VEGF represents a relevant modulator of specific events of the neurogenic process in the olfactory epithelium. Surprisingly, the information related to the presence of this receptor in the human olfactory epithelium is scarce or even absent.

We, hypothesized that VEGF may be important to modulate viability, proliferation, survival and/or migration of hNS/PCs-OE *in vitro*, cells that express neural lineage commitment markers, such nestin and βIII-tubulin proteins, but also pluripotency associated markers such as Sox2, Oct4, Klf4, Nanog and Lin28 (Franco et al., 2017; Ortiz-Lopez et al., 2017a).

2. Materials and methods

2.1. Isolation of human neural stem/progenitor cells from the olfactory epithelium

Isolation of olfactory epithelium cells was performed as described recently by us (Franco et al., 2017; Ortiz-Lopez et al., 2017a) with slight modification of the original procedure that we also reported (Benitez-King et al., 2011). The Ethical Committee of the National Institute of Psychiatry approved the study (CEI/C/084/2015-CEI/C/077/2016). The mini-international neuropsychiatric interview and Symptom Checklist-90-R were applied to evaluate participants for signs of psychopathological antecedents. Following exfoliation of the anterior region of the medial lateral turbinate and the initial part of the superior turbinate, cells contained in the brush were detached by washing with DMEM/F12 (Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 10% of fetal bovine serum (FBS; Gibco), 100UI/ml streptomycin, and 100UI/ml penicillin (Sigma, Toluca, Estado de México, México). Cells were mechanically dissociated and spun down by centrifugation (250xg for 5 min), and the cells were resuspended in DMEM/F12 supplemented with 10% FBS and antibiotics. Exfoliation performed as was mentioned above, supports that only cells of the olfactory epithelium were isolated (Franco et al., 2017). In fact, some cells obtained immediately after brushing and cultured for 48 h express markers such as Sox2, NeuroD, Pax6, βIII-tubulin and the olfactory marker protein (OMP) (Ortiz-López L; unpublished data). Thus, cells were subcultured at a clonal density of 1 cell/well in 96-well plates as we described recently (Ortiz-Lopez et al., 2017a). The cloned-cells used in this study were previously characterized for the expression of nestin, βIII-tubulin, vimentin, vinculin, the $p75^{NTR}$ receptor, Sox2 and for the expression of messenger that code for other pluripotency-associated markers, including Oct4, Klf4, Nanog and Lin28. Data reported in recent works of our group (Franco et al., 2017; Ortiz-Lopez et al., 2017a).

2.2. Immunocytochemistry

Human neural stem/progenitor cells derived from the olfactory epithelium were growth in DMEM/F12 (Gibco) supplemented with 10% FBS (Gibco) and antibiotics (Sigma) for one or two days. Then, cells were fixed with 4% para-formaldehyde (Sigma) in 1x PBS for 20 min and permeabilized with 0.05% Triton X-100 (Sigma) in PBS for 30 min. The aldehyde-free groups were reduced with 1 M glycine (Sigma) in PBS for 30 min. Primary antibodies were added and incubated overnight. The antibodies used were: rabbit anti-Ki67 (1:1000; Abcam, San Francisco, CA, USA), mouse anti-βIII-tubulin (1:500; Promega, Madison, WI, USA), mouse anti-vinculin (1:1000; Sigma), rat anti-VEGFr2 (Flk-1; 1:200; Millipore, Naucalpan, Estado de México, México). Additional cells grown on coverslips were used to stain actin filaments with rhodamine phalloidin (1:1000; Sigma). All secondary antibodies were raised in donkey and purchased from Jackson Immunoresearch (1:250, Jackson Immunoresearch, West Grove, PA, USA). Coverslips were mounted with PVA-DABCO (Ramirez-Rodriguez et al., 2009). Finally, labelling was observed using a Nikon Eclipse Ti epifluorescence microscope (Nikon, Melville, NY, USA). Images were captured with NIS-Elements software (Nikon), and the analysis was performed with ImageJ software (NIH, Bethesda, MA, USA). Experiments were performed three times in triplicates.

2.3. Western blot

Cells were lysed as we previously reported (Ramirez-Rodriguez et al., 2009) with RIPA buffer (150 mM NaCl, 10% glycerol, 0.5 mM EDTA, 0.5% Triton X-100, 1 mM phenyl-methyl-sulphonylfluoride, 25 μg/ml leupeptin, 25 μg/ml aprotinin and 1 mM sodium orthovanadate in 50 mM Tris-HCl, pH 7.6) and homogenized with an ultrasonic homogenizer. Protein content was quantified using Bradford reagent (Bio-Rad, Philadelphia, PA, USA). The separated proteins were then transferred to a nitrocellulose paper. The blots were probed with a rat anti-VEGFr2 (Flk-1) antibody (1:1000; Millipore). Protein was visualized with the Millipore enhanced chemiluminescence detection system using a Chemidoc Touch photo documenter (Bio-Rad).

2.4. Cell viability under proliferation conditions (Wst-1 and BrdU assays)

To test the effect of VEGF on the viability of hNS/PCs-OE, cells were cultured under proliferation (with 10% FBS) or differentiation (without FBS) conditions. Human NS/PCs-OE were cultured in media that contained fetal bovine serum for one-day followed by 48 h of culturing in the presence of varying concentrations of VEGF (0, 0.1, 0.5, 1, 5, 10, 50, 100, 500 ng/ml; Peprotech, Ciudad de México, México), with or without FBS. In additional experiments, AHPCs were differentiated in the presence of different concentrations of VEGF. Cell viability was assessed using the Wst-1 assay (Roche) in a multimode Glomax discover ELISA reader with a $\lambda = 630$ nm (Promega). One-hour before the end of experiments, the Wst-1 reagent or BrdU (Roche, Hague Road, IN, USA) were added to the cell culture. The metabolic activity was determined through the formation of formazan using four wells per VEGF-concentration and repeated at least thrice. Plates were read in an ELISA reader at a wavelength of 450 nm (Promega). Similarly, cell proliferation was determined using a BrdU assay (Roche). In additional experiments, hNS/PCs-OE were prelabelled with 10 mM of BrdU during 1 h to further determine survival of BrdU-labelled cells after 96 h.

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